Apoptosis and expression of apoptotic regulators in the human testis following short- and long-term anti-androgen treatment

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Apoptosis and its augmentation by androgen withdrawal is an important event in the testis. In other tissues apoptosis is regulated by genes belonging to the \textit{bcl}-2 family. However, little is known about these pathways in the human testes. Human testes were obtained from patients with prostate cancer, undergoing orchidectomy for permanent androgen ablative treatment. The patients were either untreated or had previously received short- or long-term anti-androgen therapy by cyproterone acetate or GnRH agonist (goserelin). In comparison with untreated patients, testicular testosterone concentrations were reduced by 83% in patients treated with cyproterone acetate and by 99% in patients treated with goserelin. Apoptotic cells were identified in tissue sections by in-situ end labelling of fragmented DNA. The expression of Bcl-2, Bcl-xl, Bax, p53 and poly(ADP) ribose polymerase (PARP) was demonstrated in tissue extracts by Western blotting. Apoptotic germ cells were present in the spermatogenic epithelium of untreated patients and patients who received short-term anti-androgen treatment. There were few or no apoptotic cells in the seminiferous tubules following long-term anti-androgen treatment. Following short-term treatment, the concentrations of the apoptosis-related proteins examined did not change. However, in the long-term treated testes, Bcl-xl and PARP expression declined, Bax and p53 protein concentrations were unchanged, and Bcl-2 was up-regulated. In conclusion, apoptosis occurs in spermatogenic cells of the human testis and may contribute to the regulation of germ cell populations. The apoptosis-related gene products which have been described in other tissues are present in the human testis and are modulated by androgenic stimuli.

\textit{Key words:} androgen/apoptosis/bcl-2 family/testis

\section*{Introduction}

Apoptosis is a rapid, physiologically programmed cell death (Kerr \textit{et al}., 1972; Wyllie \textit{et al}., 1980). It is characterized by nuclear and cytoplasmic condensation which occurs as the cell breaks up into membrane-bound fragments containing structurally intact organelles known as ‘apoptotic bodies’ (Kerr \textit{et al}., 1972; Wyllie \textit{et al}., 1980). These apoptotic bodies are phagocytosed by neighbouring cells and rapidly degraded so that there is no inflammation in the adjacent tissue. During apoptosis there is often a double strand, endonuclease-specific cleavage of nuclear DNA at the linker regions between nucleosomes leading to the production of oligonucleosomal fragments that are multiples of 180 DNA base pairs (Wyllie, 1980).

The survival of a cell is dependent upon the regulation of specific pro- and anti-apoptotic gene products. The involvement of the \textit{bcl}-2 gene family in apoptosis has been well documented. Bcl-2 has been shown to prolong cell survival (Vaux \textit{et al}., 1988). The \textit{bcl}-x gene has a high degree of homology to \textit{bcl}-2 and is also involved in the regulation of apoptosis (Boise \textit{et al}., 1993), with the alternatively spliced long form (\textit{bcl}-xl) being functionally similar to \textit{bcl}-2 in inhibiting apoptosis. Another member of the Bcl-2 family, Bax, was the first protein to be shown to form a regulatory dimer with Bcl-2 (Oltavi \textit{et al}., 1993). The \textit{bax} gene shows sequence homology to \textit{bcl}-2 and can block the ability of \textit{bcl}-2 to inhibit apoptosis (Oltavi \textit{et al}., 1993) thereby suggesting that Bax may promote apoptosis by functional antagonism through the formation of heterodimers with Bcl-2. The tumour suppressor protein p53 functions as a transcription factor, thereby up-regulating the transcription of pro-apoptotic genes such as \textit{bax}, and possibly repressing the transcription of survival genes such as \textit{bcl}-2 (Miyashita \textit{et al}., 1994).

Poly(ADP) ribose polymerase (PARP) is a nuclear enzyme responsible for the poly(ADP) ribosylation of chromosomal proteins and nuclear enzymes (Ohashi \textit{et al}., 1983). The formation of DNA strand breaks during apoptosis is a potent stimulus for PARP activation (Ferro and Olivera, 1982). The induction of PARP may be an attempt by the dying cell to repair DNA damage caused by nuclease activation (Ohashi \textit{et al}., 1983). PARP is a substrate for the caspases (Lazebnik \textit{et al}., 1994), in particular for one family member, caspase-3 (Tewari \textit{et al}., 1995). This attempt to repair DNA damage during apoptosis would be futile as the proteases cleave PARP into lower molecular weight fragments (Lazebnik \textit{et al}., 1994). Apoptosis occurs in many different tissues, both healthy and malignant.
and neoplastic, and it can be induced by physiologic or noxious agents. The induction of apoptosis in the spermatogenic epithelium of the testis by gonadotrophin or androgen withdrawal (Tapanainen et al., 1993; Sinha Hickim et al., 1995; Billig et al., 1995; Brinkworth et al., 1995) or selective immunoneutralization of luteinizing hormone (LH) (Marathe et al., 1995) and follicle stimulating hormone (FSH) (Shetty et al., 1996) has been reported widely in rodents. The effects of these treatments can be prevented to varying degrees by testosterone, FSH or human chorionic gonadotrophin (HCG) administration (Tapanainen et al., 1993; Sinha Hickim and Swerdloff, 1995; Marathe et al., 1995; Henriksen et al., 1996). These studies suggest that LH-stimulated testosterone production in the rodent testis is essential for the maintenance of spermatogenesis and prevention of germ cell degeneration by apoptosis.

Recently, apoptosis has also been observed in adult human seminiferous tubules cultured in vitro in the absence of testosterone (Erkkila et al., 1997). Additionally, apoptosis occurs spontaneously as observed in vivo in orchidectomized testes from men undergoing surgery for prostate cancer and in testes from men obtained at autopsy following sudden traumatic death (Brinkworth et al., 1997; Sinha Hickim et al., 1998). It has also been shown that loss of androgen resulting from cessation of HCG treatment for cryptorchidism in prepubertal life increases apoptosis in the human testis (Heiskanen et al., 1996; Dunkel et al., 1997a,b).

The aim of this study was to investigate the regulation of apoptosis in vivo in testes of adult men from whom androgen support had been withdrawn as a consequence of short or long-term anti-androgen therapies in the treatment of prostate cancer. In addition, we also investigated the expression of p53 and some of the Bcl-2 family members following androgen ablation, as little is known about the role of these proteins in the control of cell populations in the human testis.

Materials and methods

Reagents

Tween-20 and proteinase K were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Testosterone was obtained from Sigma and 1,2,6,7-[3H]testosterone was obtained from Amersham Life Sciences (Little Chalfont, Bucks, UK). The anti-testosterone rabbit polyclonal was raised against testosterone-3-CMO-BSA and was obtained from Biogenesis Ltd (Poole, Dorset, UK). The anti-Bax (1:1000) rabbit polyclonal was obtained from Pharmingen Laboratories (San Diego, CA, USA). The rabbit anti-Bcl-xl (1:500) polyclonal was obtained from Transduction Laboratories (Lexington, KY, USA). The mouse anti-Bcl-2 (1:500) monoclonal was obtained from Dako Ltd (High Wycombe, Bucks, UK). The mouse anti-p53 (1:500) monoclonal was obtained from Calbiochem Novabiochem Ltd (Beeston, Nottingham, UK). The anti-PARP mouse monoclonal was a kind gift from Dr K.Caldecott (University of Manchester, Manchester, UK). The horseradish peroxidase-conjugated secondary antibodies (anti-rabbit IgG and anti-mouse IgG) and the PVDF membranes were obtained from Amersham. The ApopTag kit for in-situ end labelling (ISEL) was supplied by Oncor Appligene (Chester-Le-Street, Durham, UK). Protein standards and protein assay reagents were obtained from BioRad Laboratories Ltd (Hemel Hempstead, Herts, UK).

Patients

Testes were obtained from 13 patients (aged 63–91 years) undergoing bilateral subcapsular orchidectomy for prostate cancer. Prior to orchidectomy, four patients (aged 70–79 years) had received short-term anti-androgen therapy with cyproterone acetate (100 mg, orally three times daily) for 10–16 days and five patients (age 79–91 years) had received long-term androgen depletion by gonadotrophin releasing hormone (GnRH) agonist (goserelin 3.6 mg s.c., monthly) for 3–48 months. The testes from four patients (aged 63–80 years) who did not receive any anti-androgen treatment prior to orchidectomy were included as controls. At surgery, the testes were immediately cooled on ice. The weights of both testes were recorded.

Radioimmunoassay

One testis from each patient was snap-frozen in liquid nitrogen and stored at –70°C until required for protein extraction (Maniatis et al., 1982) and testosterone radioimmunoassay. Combined intratesticular testosterone and dihydrotestosterone production were determined in homogenates of testicular tissue. The standard curve and samples were assayed after extraction with petroleum ether:diethyl ether (1:1) using an antibody that reacts with both testosterone (100%) and dihydrotestosterone (100%) but only shows 2.1% cross-reactivity with epi-testosterone (Morris et al., 1986). The sensitivity of the assay was 0.01 pmol per tube. Total testosterone and dihydrotestosterone synthesis in the testis extracts was expressed as pmol/g of tissue.

In-situ end labelling of DNA in apoptotic cells

One testis from each patient was fixed in Bouin’s fluid and paraffin-embedded. Apoptotic cells were identified in tissue sections (5 µm) by ISEL using an ApopTag kit (Appligene Oncor). Briefly, sections were deparaffinized in xylene and rehydrated in a graded ethanol series (100%, 95, 70 and 40%) followed by phosphate-buffered saline (PBS; 10 mM sodium phosphate dibasic, 1.8 mM potassium phosphate monobasic, 137 mM NaCl, 2.7 mM KCl, pH 7.4). The sections were digested with proteinase K (10 µg/ml) for 20 min at room temperature, washed with nanopure water (4 × 2 min) and incubated with equilibrium buffer for 5 min. Sections were incubated with terminal deoxynucleotidyl transferase reaction mixture for 1 h at 37°C in a humidified atmosphere and the reaction stopped by immersion in buffer (10 min). The sections were washed in PBS (3 × 5 min) and incubated with peroxidase-conjugated anti-digoxigenin for 30 min at room temperature in a humid atmosphere. Following washing in PBS (4 × 5 min), sections were incubated with diaminobenzidine (DAB) substrate for 10 min at room temperature, washed in nanopure water (4 × 5 min), dehydrated stepwise in ethanol (40, 70, 95 and 100%) and xylene, and mounted with DePeX (BDH, Merck Ltd, Poole, Dorset, UK). The intensity of the in-situ end labelling was graded on an arbitrary 4-point scale: 0, occasional cells with variable staining; +, weak staining in a few cells; ++, moderate staining in many cells; and ++++, many cells strongly labelled.

Western analysis

Protein determinations in the extracts were carried out using the BioRad protein assay method (Bradford, 1976). 50 µg of protein was loaded per well onto a 10% sodium dodecyl sulphate–polyacrylamide electrophoresis gel (Laemmli, 1970) and resolved at 25 mA. Protein was transferred onto Polyvinylidene fluoride (PVDF) membranes overnight (35 V). Membranes were blocked using PBS containing 0.5% Tween-20 (PBST) and 5% non-fat milk. They were probed for 2 h at room temperature using the primary antibodies indicated previously. The membranes were washed in PBST (6 × 10 min) and incubated for 1 h with the appropriate horseradish peroxidase-conjugated secondary antibody (1:5000). Following further washing.
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Figure 1. Intratesticular androgen concentration and paired testes weights. (A) The total testosterone and dihydrotestosterone concentration (in pmol/g tissue) from control patients who did not receive any treatment, patients who received short-term anti-androgen treatment with cyproterone acetate or patients who received long-term anti-androgen treatment with goserelin, prior to orchidectomy. Note that the graph is plotted on a log scale. (B) The paired testes weights for the three groups of patients. For the control and short-term treatments, the data are expressed as the mean ± SEM from four patients. For the long-term treatment, the data are expressed as the mean ± SEM from five patients. Statistically significant differences: *P < 0.05 and **P < 0.01.

(6×10 min), visualization was achieved by chemiluminescence using Amersham ECL reagents. The blots shown are representative of three control patients paired against either three short-term anti-androgen therapy patients or three long-term anti-androgen therapy patients. Quantification of band intensities was achieved using the Molecular Analyst package from BioRad. Data are presented as percentage of controls for each apoptotic protein analysed, with the control values having been assigned a value of 100%.

Statistics
For the testosterone values and protein quantification data, the results shown are the mean ± SEM. Statistical significance was determined using Students’ t-test. P-values < 0.05 and < 0.01 were considered to be significant.

Results

Intratesticular androgen concentration and paired testis weight
Figure 1A shows the total androgen concentration from the three groups of patients. The level of intratesticular androgen production in the control patients ranged from 1.83 to 6.16 pmol/g tissue (mean 3.24 ± 1.03). In comparison to the controls, the androgen concentration was significantly lower (P < 0.05) in testes exposed to short-term treatment with cyproterone acetate (range 0.02–1.70 pmol/g tissue; mean 0.57 ± 0.38). Intratesticular androgen was even lower in patients who received long-term treatment with goserelin (P < 0.01 compared to control; range 0.004–0.05 pmol/g tissue, mean 0.020 ± 0.008). Figure 1B shows that following either short- or long-term anti-androgen therapy the paired testis weight was not significantly different from the control.

Identification of apoptotic cells using in-situ end labelling
A comparison of apoptotic cell labelling in the short- and long-term treatment groups is shown in Figure 2. Occasional condensed nuclei of apoptotic germ cells were clearly identifiable in seminiferous tubules from testes of untreated control patients (data not shown). The most predominantly labelled cell types were spermatocytes and round spermatids. More extensive labelling of apoptotic cells in the testis of a patient after short-term treatment with cyproterone acetate is shown in Figure 2A. The labelled cells appeared to be in localized regions of the seminiferous tubules. Following long-term goserelin treatment, there was marked tubular atrophy with thickening of the tunica propria (Figure 2B). Spermatogenesis was arrested and there was little evidence of apoptotic germ cells.
The extent and intensity of end labelling in the three treatment groups are summarized in Table I.

### Table I. Frequency of apoptotic cells identified by in-situ end labelling in the three treatment groups

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Patient</th>
<th>Scorea</th>
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<tr>
<td>Control (no treatment)</td>
<td>1</td>
<td>++</td>
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<tr>
<td></td>
<td>2</td>
<td>+</td>
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<tr>
<td></td>
<td>3</td>
<td>0</td>
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<td></td>
<td>4</td>
<td>0</td>
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<tr>
<td>Cyproterone acetate (short-term treatment)</td>
<td>5</td>
<td>0</td>
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<tr>
<td></td>
<td>6</td>
<td>+++</td>
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<td>7</td>
<td>+</td>
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<td></td>
<td>8</td>
<td>+</td>
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<tr>
<td>Goserelin (long-term treatment)</td>
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a The intensity of the in-situ end labelling was graded on an arbitrary 4-point scale: 0, occasional cells with variable staining; +, weak staining in a few cells; ++, moderate staining in many cells; ++++, many cells strongly labelled.

The effect of short-term anti-androgen treatment on the expression of Bcl-xl, Bcl-2, Bax, PARP and p53 in the human testis

Western blot analysis revealed that Bcl-xl, Bcl-2, Bax and p53 were expressed in the human testes (figure 3). However, the expression of these proteins and the PARP protein after short-term anti-androgen treatment were not significantly different from the controls (Figure 3A; graphical representation: Figure 4A).

The effect of long-term anti-androgen treatment on the expression of Bcl-xl, Bcl-2, Bax, PARP and p53 in the human testis

In comparison to the controls, long-term treatment with goserelin resulted in a statistically significant (P < 0.01) decrease in the expression of Bcl-xl and PARP (Figure 3B; graphical representation: Figure 4B). In contrast, Bcl-2 expression after long-term anti-androgen treatment was significantly greater than in the controls (P < 0.01) whereas the expression of Bax and p53 was unchanged.

Discussion

Our objective was to document the presence of the major apoptotic gene products in the human testis and to see if the expression of these proteins is altered when degenerative apoptotic changes are induced by withdrawal of androgen. Short-term administration of cyproterone acetate resulted in a substantial reduction of testicular androgen production. Cyproterone acetate is an antagonist of androgens acting at the receptor (reviewed in Labrie et al., 1996) and has also been shown to reduce serum testosterone concentrations both in rats and in men (Knuth et al., 1984; Goncalvo et al., 1993), as well as in hyperandrogenic states in women (Grumwald et al., 1994; Grigoriou et al., 1996; Grisaru et al., 1996). The latter effect is due to the action of cyproterone acetate (particularly at high doses such as those used in our study) acting as a progestagen at the hypothalamus to inhibit the release of GnRH (Couzinet et al., 1986). Cyproterone acetate also acts on the Leydig cell by inhibiting 3β-hydroxysteroid dehydrogenase and C17,20-lyase (Honma et al., 1995). Our observation that intratesticular testosterone is reduced by cyproterone acetate is consistent with the anti-androgenic actions of this drug. Additionally we showed that long-term administration of goserelin to the prostate cancer patients results in a profound suppression of intratesticular androgen production. Goserelin is a GnRH agonist which induces suppression of androgen production by GnRH receptor desensitization (Labrie et al., 1980; Faure et al., 1982). Goserelin has also been reported to act at the steroidogenic level by decreasing 3β-hydroxysteroid dehydrogenase, 17α-hydroxylase and C17,20-lyase activities (Petersson et al., 1987). The results of our present study confirmed previous studies which showed that GnRH agonists reduced serum androgen concentrations to below castrate levels after 3-4 weeks of treatment (Ayub and Levell, 1990; Vogelzang et al., 1995; Debruyne et al., 1996; Fernandez del Moral et al., 1996) and that intratesticular testosterone production is reduced in...
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Figure 4. Quantitative analyses of Bcl-xl, Bcl-2, Bax, poly(ADP) ribose polymerase (PARP) and p53 expression following short- and long-term anti-androgen treatment. Quantitative densitometric analyses of protein expression determined from Western blots of control patients and patients who have undergone (A) short-term anti-androgen treatment with cyproterone acetate or (B) long-term anti-androgen treatment with goserelin prior to orchidectomy. For the control and short-term treatments, the data are presented as the mean ± SEM of four patients. For the long-term treatment, data are presented as the mean ± SEM of five patients. The data are presented as percentage of controls where control values have been assigned a value of 100%. O.D. = optical density. Statistically significant difference: **P < 0.01.

In this study, we have reported that apoptotic germ cells are present in the testes from patients who have received no treatment prior to orchidectomy. These findings are in agreement with those from recent studies (Brinkworth et al., 1997; Sinha Hikim et al., 1998). In the normal rat testis it has been suggested that cell death accounts for the loss of up to 75% of germ cells before they reach maturity (Huckins, 1978). This spontaneous loss may be a consequence of limitations in the capacity of the Sertoli cells with regard to the number of developing germ cells it can support. As a result, the remainder die by apoptosis and only 25% develop into mature sperm.

We predicted that our results would show that even against this background of spontaneous cell death, there would be an increase in the number of apoptotic germ cells following short-term androgen withdrawal in the ageing human testis. However, we were not able to show this convincingly. These data contrast with the findings from in vivo studies in the rat (Tapanainen et al., 1993; Sinha Hikim et al., 1995; Billig et al., 1995; Brinkworth et al., 1995). During androgen withdrawal-induced apoptosis in the rat seminiferous tubules, the prominent cell types undergoing programmed cell death are spermatocytes (Billig et al., 1995; Sinha Hikim et al., 1995). However, our results and those of others (Brinkworth et al., 1997; Sinha Hikim et al., 1998) show that spontaneous apoptosis occurs in a wider range of germ cell types in the human testis in vivo. It is possible that in our study, an increase in apoptosis following short-term anti-androgen treatment might be detectable if the period of treatment with cyproterone acetate could be extended beyond 3 weeks. Although intratesticular androgen concentrations declined after 10–16 days of cyproterone acetate administration, the concentration may still be high enough to prevent more widespread apoptosis. This is supported by the findings of others (Brinkworth et al., 1997) who have shown that there is no significant difference in the level of apoptosis between patients who have received no treatment prior to orchidectomy and those who had undergone short-term therapy with cyproterone acetate for periods of up to 7 days. When gonadotrophin is suppressed by exogenous testosterone in man for hormonal male contraception, the maximum suppression of sperm production occurs only after 12–16 weeks (Anderson et al., 1997). Furthermore, it may be difficult to discern a marked increase in cell death following short-term cyproterone acetate treatment against the background level of apoptosis in the control testes from ageing (mean age 71 years) men. In the human, stages of spermatogenesis are organized in a spiral arrangement along the seminiferous tubule such that several stages can be seen in any one cross-section. This arrangement may account for the more patchy distribution of apoptosis in the seminiferous tubules of our short-term-treated patients as compared to that observed in the rat testis. Following long-term treatment with GnRH agonist, we observed little or no germ cell apoptosis even though testosterone was very low. In these chronically androgen-depleted testes, any apoptotic degenerating germ cells would have already been scavenged, and mitotic or meiotic division of spermatocytes is likely to have been arrested.

Despite the marked fall in androgen support to the spermatogenic epithelium following short-term anti-androgen treatment, there were no detectable changes in p53, Bcl-2, Bcl-xl, Bax nor PARP concentrations. This is consistent with our earlier study which showed that the expression of p53 and the Bcl-2 family members were unchanged in the rat testis following short-term androgen withdrawal with ethane dimethanesulphonate (Woolveridge et al., 1997). This may reflect the fleeting nature of changes in the expression of genes which trigger the cascade of downstream events leading to programmed cell death. These transient and early changes in gene regulation may only be detectable with carefully timed sampling which is not possible in a clinical study of this nature. It is also possible that other genes are important in germ cell apoptosis. The mcl-1 gene, a member of the bcl-2 family, protects against programmed cell death, although Mcl-1 immunostaining is only seen in the Leydig cells of the human testis (Krajewski et al., 1995). The bak gene, another
family member has been shown to be pro-apoptotic. Bak protein has been immunolocalized exclusively to the Sertoli and Leydig cells of the human testis (Krajewski et al., 1996). Furthermore, the bcl-2 family members are thought to regulate apoptosis by the formation of hetero- and homodimers and the prevailing outcome depends on the ratio of protector to promoter. With this in mind, it is possible that the changes which are important after androgen withdrawal are not in the absolute concentrations of these gene products, but in protein–protein interactions and relative ratios of Bcl-2 family proteins bound to one another.

In the long-term androgen-depleted testes, Bcl-xL and PARP protein concentrations declined. This could be explained by their known roles in cell survival and DNA repair leading to the expectation that both are suppressed during apoptosis. However, in the testis chronically depleted of testosterone an alternative explanation for this decline in expression is the loss of specific cell types expressing such genes. For example, Bcl-xL has been immunolocalized to the spermatocytes and spermatids of the human testis (Krajewski et al., 1994b) and these are the cell types that have undergone apoptosis in response to androgen withdrawal. However, Bcl-2 has also been localized exclusively to the spermatids in the normal human testis (Krajewski et al., 1995) but the expression of this gene product in the testes of the long-term-treated patients is actually up-regulated. When gene expression does not decline following the loss of cell types known to express such genes, there is possibly a compensatory increase in expression of these genes in remaining cell types in the testes. Since spermatids are absent in our chronically treated testes, it is possible that the up-regulation of Bcl-2 occurs in other cells such as the Sertoli cells or spermatogonia. Bax expression was not altered by long-term androgen withdrawal. In the mouse testis, Bax has been immunolocalized to the germinal cells located near the basement membrane but not in more differentiated cell types (Krajewski et al., 1994a). If Bax is localized to the same cells in the human testis, it may well be that the lack of any discernible change in expression of this protein could be accounted for by the maintenance of spermatogonia whose survival is not androgen dependent.

In conclusion, apoptosis occurs in the human testis and may contribute to the control of cell populations in the spermatogenic epithelium. However, observations of apoptosis in the human testis are complicated by the presence of many different cell types, both somatic and germinal, maintained in a complex architectural arrangement. Even though the classical apoptosis regulators, Bcl-2, Bax and Bcl-xL are unchanged following 10–16 days of anti-androgen treatment, they may still play a role at other time points following androgen withdrawal. Further studies are aimed at determining the involvement and cellular localization of these gene products following androgen withdrawal.

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References


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